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Human D^{IIIa} Erythrocytes: RhD Protein is Associated With Multiple Dispersed Amino Acid Variations

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As a partial D antigen of the Rh blood group system, the D category IIIa phenotype occurs mainly in Blacks, but its molecular basis has not been defined. Here we describe studies of the D category D^{IIIa} and VS+ red blood cells (RBC) from two unrelated probands by Southern blot, cDNA PCR, and nucleotide sequencing. Rh haplotyping by *Sph* I restriction fragment length polymorphisms indicated that the two probands carried *Dce/dCe* and *Dce/DcE* genotypes, respectively. Sequence analysis of Rh cDNAs showed that their erythroid cells expressed both *D* and *CE* transcripts. Nevertheless, the *D* transcripts were found to contain four nucleotide changes scattered in three exons: nt455 A-to-C (exon 3), nt602 C-to-G (exon 4), nt 654 C-to-G (exon 5), and nt667 T-to-G (exon 5). These variations resulted in the following amino acid substitutions characteristic of RhCE polypeptides: 152 Asn-to-Thr, 201 Thr-to-Arg, 218 Ile-to-Met, and 223 Phe-to-Val. The 152Thr and 223Val residues were predicted to reside in proximity to the third and fourth extracellular loops, respectively. Together, these results establish a correlation of the four amino acid changes in the RhD protein with the expression of D^{IIIa} as a partial D antigen on the RBC membrane. Since the varied nucleotides identified in D^{IIIa} all pre-exist in *CE,* they are likely to have originated from *CE* by templated micro-conversion event(s). The identification of a specific nt736 C-to-G transversion in *CE* in the two probands suggests that 245Val may involve the expression of VS antigen. Am. J. Hematol. 55:139–145, 1997.

Key words: Rh blood group system; partial D antigen; RBC membrane proteins; Rh locus; genetic variation

INTRODUCTION

The Rh blood group system in man is clinically important because of the extreme immunogenicity of its D antigen and the high incidence of Rh-negative phenotypes (i.e., the absence of D antigen) in the population [1]. Another clinically significant phenomenon related to D antigen expression is the partial D status associated with both qualitative and quantitative polymorphism. As shown by serologic studies, this phenotypic diversity can be classified into six major categories (i.e., II to VII) with further subdivisions [2].

Recent studies have led to the characterization of a number of Rh genetic variants defined as partial D antigen categories [3–9]. Comparison of these variants at the molecular level indicates that exchange of homologous domains between *RHD* and *RHCE* is an important mechanism causing partial D expression. The loss or gain of a given D epitope(s) appears to depend not only on the extent of sequence exchange but also on the context into which the exchanged sequence is placed. These correlations explain some puzzling observations on partial D variants and suggest that both linear sequences and proper conformation are required for D antigen presentation.

Of partial D variants, D category III is divided into three subgroups referred to as a, b, and c. D^{IIIa} and D^{IIIb} occur mainly in Blacks and D^{IIIc} in Whites [2]. Molecular analysis showed that D^{IIIb} and D^{IIIc} are each associated

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with a RhD-CE-D hybrid gene; the former has gained its exon 2 from *RHCE* [5], whereas the latter has gained its exon 3 from *RHCE* [7]. During the course of studying Rh genetic variations in Blacks, we identified two unrelated individuals whose red blood cells (RBC) exhibit both D^{IIIa} and VS phenotypes. A detailed analysis of gene structure and transcript expression showed that both *RHD* and *RHCE* in the two probands carry specific nucleotide changes that are correlated with phenotypic variations. Interestingly, in contrast to D^{IIIb} and D^{IIIc}, the D^{IIIa} gene is not typified by transfer of whole exons but contains multiple nucleotide changes dispersed in three exons that pre-exist in the *RHCE* gene.

MATERIALS AND METHODS

Blood Samples

Peripheral whole blood samples were obtained from human blood donors and two Black probands. Their Rh types were as follows: Rh-positive control (H.T.), D+C+E−c−e+G+VS−; Rh-negative control (F.D.), D−C−E−c+e+G−VS−; Proband (B.P.), $D^{III a}$+C+^{W}E−c+e+G+VS+; Proband (D.J.), D^{IIIa}+C−E+c+e+G+VS+.

Isolation of Nucleic Acids and Southern Blot Analysis

Total RNA was isolated from RBC hemolysate and genomic DNA from leukocyte pellet, using the differential cell lysis method [10]. Following acetic acid titration, the RNA pellet was extracted with Trizol reagent (GIBCO-BRL, Gaithersburg, MD). Genomic DNAs were digested with *Sph* I and analyzed by Southern blot hybridization with three Rh cDNA probes [11]. These probes span exons 1–3, 4–7, and 8–10, respectively.

Synthesis and Amplification of Rh cDNAs by Reverse Transcriptase-PCR (RT-PCR)

Rh mRNAs were converted into single-stranded cDNAs by the AMV reverse transcriptase and then amplified by PCR, as previously described [12]. The strategy took advantage of the difference between *RHD* and *RHCE* in the nonhomologous 3′-untranslated (3′-UT) regions, thus allowing the synthesis and amplification of *D* and *CE* transcripts to proceed separately. The *D*(3′) primer for RhD cDNA synthesis was 5′-GTATTCTACAGTGCATAATAAATGGTG-3′ (antisense, nt 1,432–1,458), whereas the *CE*(3′) primer for RhCcEe cDNAs was 5′-CTGTCTCTGACCTT-GTTTCATTATAC-3′ (antisense, nt 1,363–1,388). After cDNA synthesis at 42°C for 75 min, the reverse transcriptase was inactivated at 72°C for 10 min. From this cDNA reaction, 2.5 μl were taken, added to a 50-μl premade PCR mixture, and amplified for 35 cycles. The conditions for amplification were as described [12] and the primers were: set Ia, 5′-ATGAGCTCTAAGTAC-CCGC GGTCTG-3′ (sense, nt 1–25 of exon 1), and lb, 5′-TGGCCAGAACATCCACAAGAAGAG-3′ (antisense, nt 640–663 of exon 5); set IIa, 5′-CCAAA-ATAGGCTGCGAACACGTAGA-3′ (sense, nt 515–539 of exon 4), and IIb, 5′-TTAAAATCCAACAGC-CAAATGAGGAAA-3′ (antisense, nt 1,228–1,254 of exon 10).

Amplification of RH Genomic Sequences

RH genomic sequences encompassing exons 4 and 5 were amplified by PCR using the thermostable *Taq* DNA polymerase [13]. PCR was performed for 30 cycles in 50 μl of reaction volume containing the forward (*F*) and reverse (*R*) primers: *F*1, 5′-ATTGCAGACAGACTAC-CACATGAAC-3′ (sense, intron 3/exon 4 junction); *F*2, 5′-GACGTGACTTCCCCATCTAACTCT-3′ (sense, 3′ end of intron 4); and *R*, 5′-GCTGATCTTCCT(C)T-TGGGGGTGAGC-3′ (antisense, nt 775–798 of exon 5). The time and temperature segments for cycling were programmed as follows: DNA denaturation, 94°C for 60 sec; primer annealing, 55°C for 45 sec; and chain extension at 72°C, 60 sec for F1-R primer pair and 30 sec for F2-R primer pair. The final chain extension step was at 72°C for 7 min.

Direct Nucleotide Determination by PCR Cycle Sequencing

Amplified *RH* cDNA and genomic DNA products were purified by native 5% polyacrylamide gel electrophoresis (PAGE) and eluted in 1 × TE buffer. The recovered DNA templates were directly cycle-sequenced on a 373A automated DNA sequencer (Applied Biosystems, Foster City, CA) using fluorescent dye tags as chain terminators. The deduced primary sequence was analyzed by hydropathy plot to assess the arrangement of putative membrane segments [14].

RESULTS

Southern Blot Analysis and Rh Haplotyping

To genotype the two probands and determine whether their *RH* loci harbored any gross alterations, genomic DNAs were digested with restriction enzymes and analyzed by Southern blot. In *Hin*d III digestion, no apparent size difference was noted, except for a reduced intensity in *RHD* bands in B.P. (Fig. 1, *Hin*d III panel). In *Sph* I digestion, the two probands showed, in the polymorphic region encompassing exons 4–7 [15], different banding patterns (Fig. 1, *Sph* I panel) that correlated with their respective genotypes (Fig. 1, right). In B.P., the presence of 1.2-, 1.9-, 5.4-, and 8.9-kb bands in association with a dosage decrease in *D*-specific 1.2- and 8.9-kb bands indicated a most likely genotype, *Dce/dCe*. In D.J., the presence of 1.2-, 1.9-, 5.4-, 7.3-, and 8.9-kb bands with-

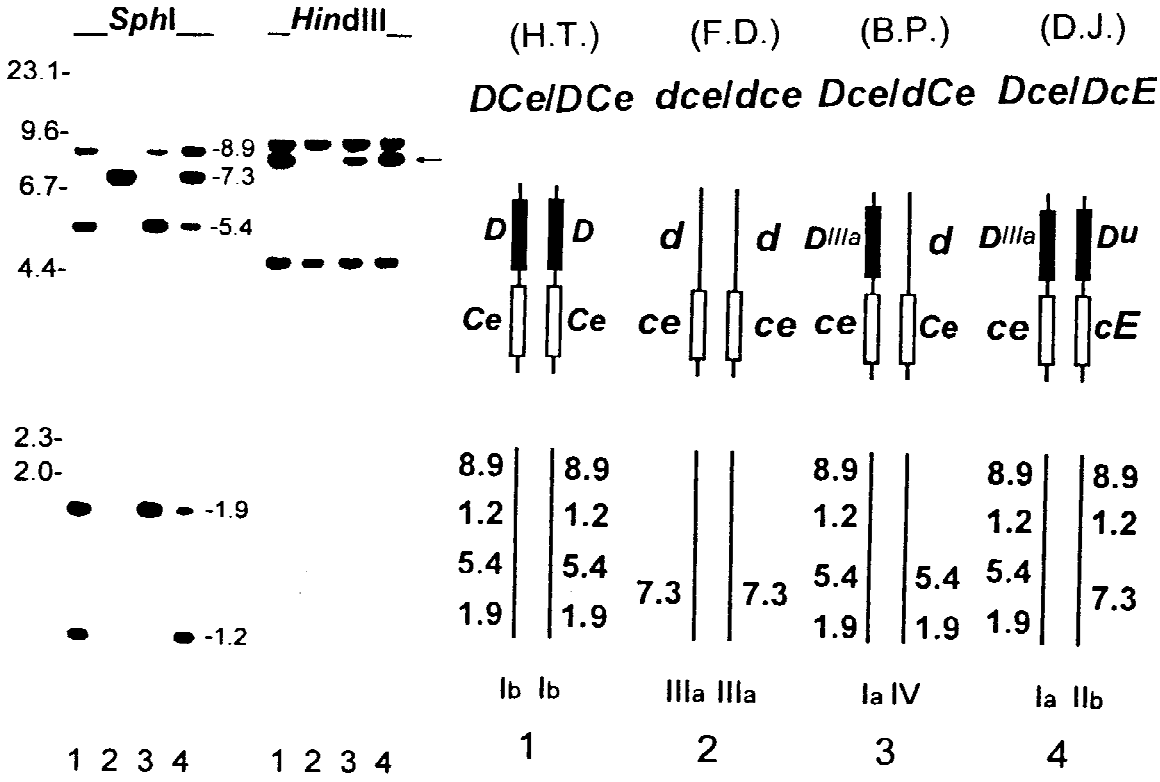

Fig. 1. Southern blot and Rh genotyping of the two probands with D^{IIIa} and VS phenotypes. Left: *Sph* I and *Hind* III genomic blots hybridized with the Rh cDNA probe encompassing exons 4 through 7. Designations are: 1, RhD-positive (HT); 2, RhD-negative (FD); 3, D^{IIIa} proband (BP); and 4, D^{IIIa} proband (D.J.). The size of λ DNA *Hind* III markers is denoted at the left margin. The size of five *Sph* I bands also is indicated. Note that B.P. shows a reduced intensity in *RHD* bands on both *Sph* I (1.2- and 8.9-kb bands) and *Hind* III blots (arrow). No other alteration was detected except for a specific 5′ *Sph* I marker linked to D^u in proband D.J. (gel not shown). Right: Possible genotypes of the two probands as opposed to those of control individuals. The vertical bars denote *D* (solid) and *CE* (open, including *Ce, ce,* and *cE* alleles) of *RH* locus. A straight line with *d* indicates the D-negative status lacking the *D* gene, whereas D^u illustrates the suppressed form of *D* in D.J. Below the diagram of each genotype is also shown the combination of *Sph* I RFLPs that define the haplotype frameworks (HF): Ib, IIIa, Ia, IV, and IIb (see reference [15] for details).

out reduction of *D* dosage indicated a most likely genotype, *Dce/DcE*. As no apparent gross alteration was noted, the molecular basis for the D^{IIIa} and VS phenotypes might involve subtle changes in the *RH* genes. In D.J., the *D* gene in trans to D^{IIIa} most likely occurred as a nondeletion form of weak D or D^u, as genomic DNA was linked to a specific marker in the 5′ region (data not shown).

Characterization of Rh Transcripts Expressed in D^{IIIa}+VS+ Erythroid Cells

To define the underlying structural alteration, the *RHD* or *CE* cDNA was obtained by RT-PCR of total erythroid RNA isolated from the two probands. Both *D* and *CE* transcripts were found to be expressed and were comparable to those of controls in size (data not shown). These cDNA forms were then cycle-sequenced after gel purification and were found to maintain the same open reading frame as the normal genes encoding 417 amino acids [16–18]. However, the *RHD* cDNA from B.P. contained four nucleotide changes that were dispersed in three exons but pre-existed in the *RHCE* gene. These substitutions occurred at the following positions according to their distance from the first base of initiation codon ATG: nt 455 A-to-C in exon 3, nt 602 C-to-G in exon 4, nt 654 C-to-G in exon 5, and nt 667 T-to-G in exon 5 (Fig. 2). These variations were predicted to result in four amino acid changes characteristic of the CcEe Rh polypeptides: 152 Asn-to-Thr, 201 Thr-to-Arg, 218 Ile-to-Met, and 223 Phe-to-Val. (Fig. 2). Analysis of *RHD* cDNA sequences from D.J. showed that only these four positions were heteroplasmic for both D^{IIIa} and *D* bases, albeit the signals for the *D*-specific bases were significantly reduced probably due to a weak expression of D^u. Together, the results confirmed that B.P. was hemizygous, while D.J. was heterozygous, for the D^{IIIa} gene.

Analysis of *RHCE* transcripts from the two probands by restriction digestion and DNA sequencing showed a correlation of the primary structure with their CcEe phenotypes. However, the *CE* transcripts of both probands

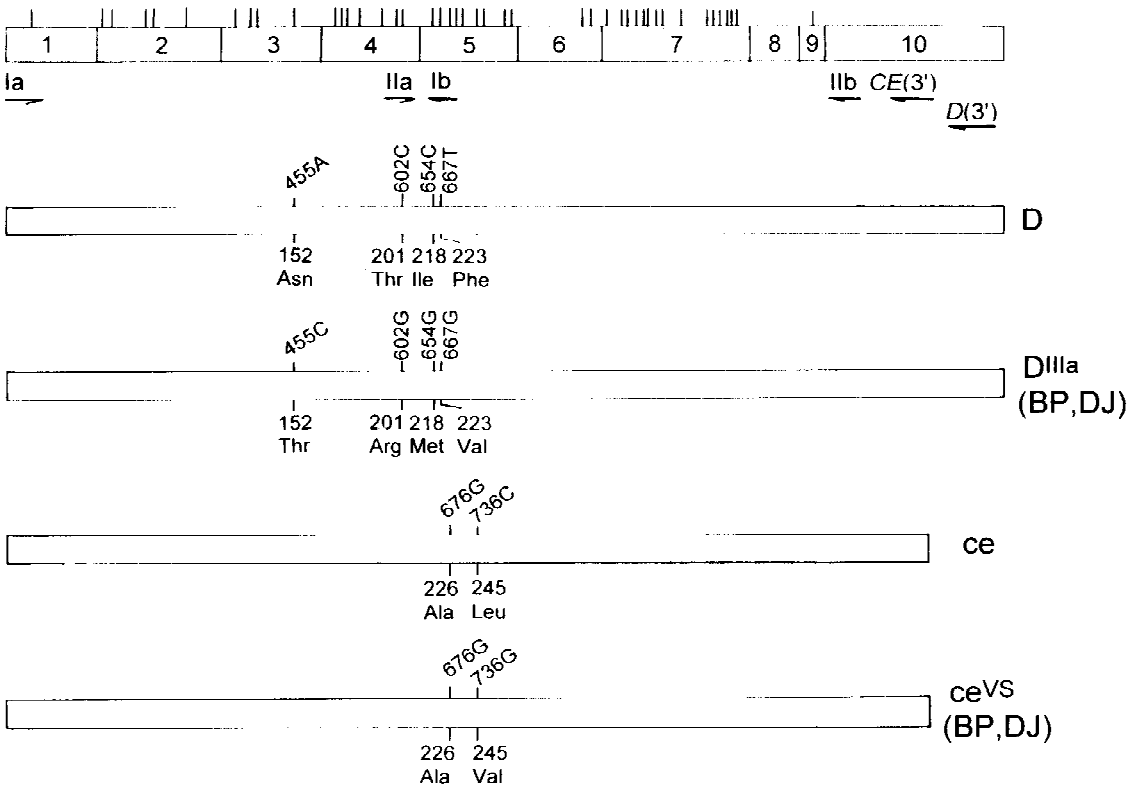

Fig. 2. Structure of Rh transcripts and predicted amino acid changes in the Rh proteins. Top: Diagram of Rh cDNA structure with ten exons. Nucleotide differences between *D* and *CE* and their distribution in individual exons are indicated by vertical thin lines. The location of various primers used in cDNA synthesis and amplification is shown. Bottom: Diagram of D^{IIIa} and ce^{VS} transcripts identified in probands B.P. and D.J. In comparison, the *D* and *ce* transcripts are also shown. The relevant nucleotide change and its predicted amino acid substitution at a given position are indicated. Note that four changes are seen in D^{IIIa}, all of which pre-exist in *CE*, whereas only one change, i.e., C736G for Leu245Val is associated with ce^{VS} in the two probands. Leu245Val is in proximity to Ala226Pro, the site of the unique polymorphism for e/E. Note that B.P. also has a Ce^{VS} transcript (not drawn), as she is homozygous for the variation.

carried a unique nucleotide transversion, nt 736 C-to-G (codon 245), leading to a Leu-to-Val replacement (Fig. 2). B.P. was homozygous for this change because her *Ce* and *ce* transcripts both contained 736G, whereas D.J. was a heterozygote because the C and G bases coexisted in his *CE* transcripts. Notably, the G base pre-exists in the *RHD* gene [18]. Since no other specific changes were found in *CE* transcripts, the C732G or Leu245Val substitution might be correlated with the expression of VS antigen in these two individuals.

Confirmation of Nucleotide Changes in the RH Genes

Both genomic mapping and transcript analysis showed that B.P. was hemizygous for the D^{IIIa} gene, because her normal *RHD* on the homologous chromosome was absent. In addition, it has been shown that *RHD* lacks, while *RHCE* contains, the insertion sequence with an *Alu* repeat in intron 4 [12,19]. By taking advantage of these features, a specific and separate amplification of the genomic regions encompassing exons 4 and 5 of the D^{IIIa} and *CE* genes was achieved with the *F1-R* primer pair (Fig. 3A). As shown in Figure 3B, the size of the amplified D^{IIIa} or *D* fragment was smaller than that of *CE*. Sequencing of the smaller genomic fragment showed the occurrence of the three nucleotide variations (602G, 654G, and 667G) in the D^{IIIa} gene, whereas sequencing of the larger one showed the presence of 736G in the *CE* gene. As the 736G substitution eliminates a *Bfa*I restriction site in exon 5, secondary PCR of the larger *CE* and smaller *D* fragments was performed using the *F2-R* primer pair and the corresponding products were digested with the enzyme. This diagnostic test directly demonstrated the 736G homozygosity in B.P. and its heterozygosity in D.J. (Fig. 3C). These results together confirmed the above cDNA sequence analysis and established a linkage of D^{IIIa} and *VS* in the following possible configuration: $D^{IIIa}ce^{VS}/dCe^{VS}$ for B.P. and $D^{IIIa}ce^{VS}/D^{u}cE$ for D.J.

DISCUSSION

In this report, we have presented molecular data on two probands whose RBCs exhibit compound Rh anti-

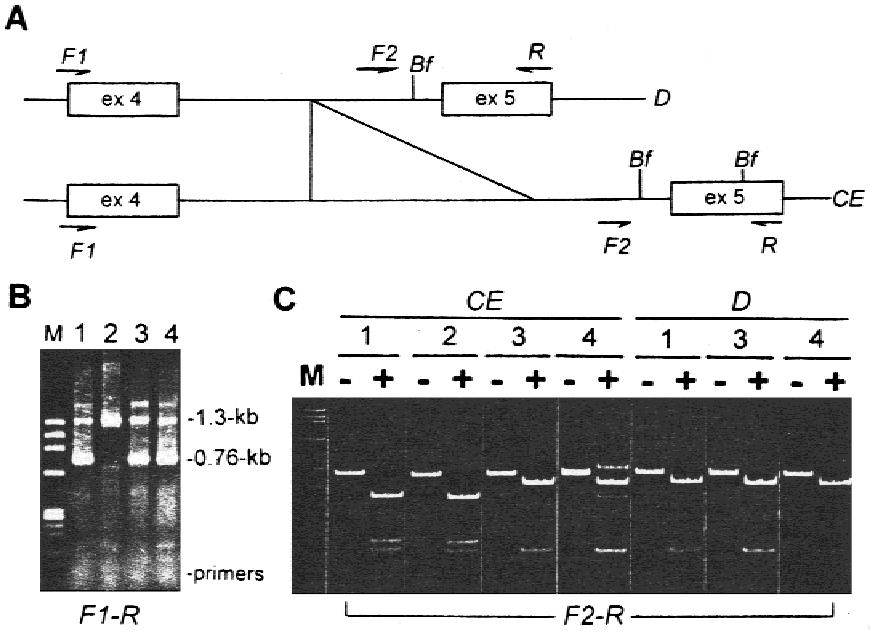

Fig. 3. Amplification and analysis of *RHD* and *CE* genomic sequences spanning exons 4 and 5. A: The *RHD* or *CE* region amplified is diagrammed. The location of primers and *Bfa* I (Bf) sites in the *F2-R* region is shown. The insertion sequence containing an *Alu* repeat in *CE* intron 4, but not in *D* intron 4 [12], is covered by a triangle. B: One point eight percent agarose gel electrophoresis of *F1-R* segments visualized by ethidium bromide. M denotes *Hae*III-digested ϕX174 DNA markers. Designations are as in Figure 1. The 1.3- or 0.76-kb band is *CE*- or *D*-specific and the latter is absent in D-negative (lane 2). A minor band above 1.3 kb may be the artifactual 0.76-kb dimer. C: Seven percent PAGE analysis of *Bfa* I-digested *F2-R* segments. The *CE* and *D F2-R* segments were amplified from gel-purified 1.3- and 0.76-kb *F1-R* bands, respectively. (−): undigested; (+): *Bfa* I-digested. Like the *D*-specific *F2-R* bands, the *CE* band of B.P. (*CE* panel 3) lacked the exon 5 *Bfa* I site, whereas that of D.J. was heteroplasmic for the site (*CE* panel 4; note an incomplete digestion).

genic variants, D^{IIIa} and VS. Both phenotypes are known to occur predominantly in Black populations. We provided evidence for the linkage of *D^{IIIa}* and *VS* in these two individuals by demonstrating that specific nucleotide changes are associated with *RHD* and *RHCE*, respectively. Nevertheless, it should be pointed out that not all D^{IIIa} RBCs coexpress the VS antigen or vice versa, conforming to an independent segregation of the two genetic entities. In the existing probands, the possibility that coexpression of the two traits might result from an unequal crossover event between the two homologous *RH* loci bearing *D^{IIIa}* and *VS*, respectively, cannot be excluded.

Concerning D^{IIIa}, its association with four specific amino acid changes in the D protein defines a partial D antigen. Assessment of possible membranous arrangement of the deduced D^{IIIa} polypeptide suggests that 152Thr and 223Val reside in proximity to the third and fourth extracellular loops, respectively (Fig. 4). In contrast, 201Arg would be close to the cytoplasmic side, whereas 218Met would remain embedded in the membrane. It is likely that 152Thr and 223Val of the D^{IIIa} protein affect the positioning of their nearby surface loops and thus alter the conformation of a given D epitope(s). Such a conformational change in D^{IIIa} protein is anticipated to be less dramatic than that in D^{VI} protein, since the latter contains a much longer RhCE sequence [3,9]. Also notably, the amino acid changes of D^{IIIa} all pre-exist at the corresponding positions of RhCE polypeptides and two of them, 152Thr and 223Val, have been noted to occur in the partial D variants, D^{IVa} and D^{Va}, respectively [4]. The dispersion of the four changes in three exons of D^{IIIa} makes a sharp contrast with D^{IIIb} and D^{IIIc} whose replacements are each confined to a single exon [5,7]. Phenotypically, the main feature of D^{IIIb} appears as loss of the G antigen due to an exchange of exon 2 [5], whereas in D^{IIIc} the site of some unknown D epitopes encoded by exon 3 is implicated [7]. Thus, despite the same category designation, the D^{III} variants are similar neither genetically nor structurally.

Genomic and transcript analyses of *RHCE* showed that one proband is homozygous, while the other heterozygous, for the C736G change in exon 5 causing a Leu245Val substitution. As no other apparent changes were found in *CE*, this particular variation leads to a correlation with the expression of VS antigen. Generally, VS travels with e^s antigen but segregates with hr^B antigen [20], a pattern with which the results of Rh phenotyping of the two probands coincide. Nevertheless, more studies will be required to define the nature of VS and its relationship with hr^B, as such a genetic correlation is not consistently observed in all examples analyzed (our unpublished data).

The finding of four *CE*-specific nucleotides dispersed in three exons of the *D^{IIIa}* gene raises the question as to how these replacements might originate. The simultaneous occurrence of multiple spontaneous point mutations as such, if not possible, would be an extremely rare event for a given structural gene. Furthermore, it should be noted that although the *CE*-specific nucleotides associated with *D^{IIIa}* are scattered, they are all flanked by

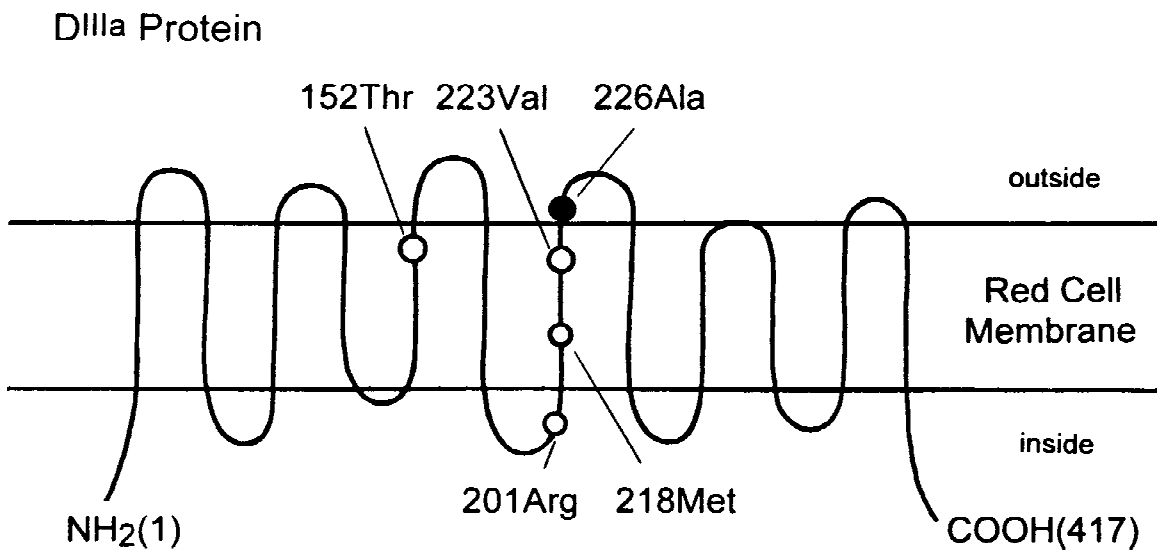

Fig. 4. A topologic model for the arrangement of transmembrane segments of the D^{IIIa} protein. The D^{IIIa} protein is predicted to contain 417 amino acids as RhD, but differs from the latter at four positions on the polypeptide backbone [18]. Of these amino acid changes (open circles), 152Thr and 223Val would be close to the third and fourth extracellular loops and thus could play an important part in displaying the D^{IIIa} phenotype. The other two would be less crucial, since 201 Arg is likely to face the cytoplasmic space and 218Met remains embedded in the membrane. As a reference, 226Ala (solid circle) on the fourth extracellular loop is indicated.

RHD-specific nucleotides. Such a mosaic arrangement suggests a possibility that they originated from a templated transfer or microgene conversion involving ''patches'' of *RHCE* sequence. This type of recombination has been implicated as an important mechanism to diversify the immunoglobulin and HLA genes [21,22]. A more complete cataloging of Rh genetic variations should unveil the significance of this mechanism to the Rh blood group system.

CONCLUSIONS

The expression of D^{IIIa} as a partial D antigen on the RBC membrane is correlated with four amino acid changes in the RhD protein. The occurrence of these RhCE-specific amino acid residues in the context of D may alter the conformation of the two nearby surface loops, thus affecting or weakening the exhibition of some D epitopes. D^{IIIa} are neither genetically nor structurally similar to D^{IIIb} and D^{IIIc}, although they are grouped in the same category. The genetic nature of VS antigen is partly related to the C736G or Leu245Val substitution, but the molecular relationship between VS and hr^B requires further investigation.

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